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Brief Definitive Report

DC-SIGN Is the Major *Mycobacterium tuberculosis* Receptor on Human Dendritic Cells

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Abstract

Early interactions between lung dendritic cells (LDCs) and *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, are thought to be critical for mounting a protective anti-mycobacterial immune response and for determining the outcome of infection. However, these interactions are poorly understood, at least at the molecular level. Here we show that *M. tuberculosis* enters human monocyte-derived DCs after binding to the recently identified lectin DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN). By contrast, complement receptor (CR)3 and mannose receptor (MR), which are the main *M. tuberculosis* receptors on macrophages (Mϕs), appeared to play a minor role, if any, in mycobacterial binding to DCs. The mycobacteria-specific lipoglycan lipoarabinomannan (LAM) was identified as a key ligand of DC-SIGN. Freshly isolated human LDCs were found to express DC-SIGN, and *M. tuberculosis*–derived material was detected in $CD14^{-}HLA\text{-}DR^{+}DC\text{-}SIGN^{+}$ cells in lymph nodes (LNs) from patients with tuberculosis. Thus, as for human immunodeficiency virus (HIV), which is captured by the same receptor, DC-SIGN–mediated entry of *M. tuberculosis* in DCs in vivo is likely to influence bacterial persistence and host immunity.

Key words: Mycobacteria • tuberculosis • dendritic cell • DC-SIGN • lipoarabinomannan

Introduction

M. tuberculosis infections are responsible for 1.5 to 2 million deaths annually. Such a dramatic situation is due, at least in part, to the ability of the airborne bacillus to resist killing by, and to parasitize host alveolar macrophages (Mϕs; reference 1). Protective anti-mycobacterial immune response involves mainly T lymphocytes that activate the Mϕ microbicidal functions through the release of interferon γ (2, 3). Priming of naive T lymphocytes against mycobacterial antigens is thought to occur in the proximal LNs and to rely on a particular subset of phagocytic cells, the dendritic cells (DCs). Indeed, DCs exhibit the unique ability to activate naive lymphocytes after migration from infection sites, where they capture antigens, to the LNs where they express high amounts of presentation molecules, such as MHC-II, and costimulatory molecules, such as CD80 and CD86 (4). The early interaction between the DCs present as a dense network in the airway mucosa (5) and *M. tuberculosis* is thus likely to be critical for mounting a protective anti-mycobacterial immune response (3, 6–9). However, *M. tuberculosis* interactions with DCs are poorly understood at the molecular level. In particular, the ability of *M. tuberculosis* to replicate in DCs, relative to Mϕs, remains controversial (7, 9, 10), and the receptor(s) used by *M. tuberculosis* to bind and to enter DCs are still unknown, whereas those involved in the parasitism of Mϕs have been well characterized in vitro. Mycobacterial binding to Mϕs occurs in cholesterol-rich domains of the host cell plasma membrane (11) and involves CR3, together with other molecules like MR, CR1, CR4, CD14, surfactant protein (SP)-A receptors, as well as scavenger receptors (12, 13). Other surface

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molecules, such as Toll-like receptors (TLRs), are also essential for mycobacterial interactions with phagocytic cells (14), though their role in mycobacterial entry remains to be evaluated. Some of these receptors (e.g., CR3, MR) are present on DCs and may be involved in the binding and entry of mycobacteria into these cells. However, DCs express additional receptors that are dedicated to capture of antigens. These additional receptors include the recently identified DC-SIGN (15), a calcium-dependent (C-type) lectin, containing a carbohydrate recognition domain (CRD) at its extracellular COOH-terminal end, that recognizes mannose-rich molecules (16). DC-SIGN was initially described as a receptor for ICAM-3 at the surface of T cells, triggering the formation of the immunological synapse between DCs and naive T lymphocytes. Interestingly, DC-SIGN binds to HIV and simian immunodeficiency viruses, and is involved in the trans-infection of $CD4^+$ T lymphocytes by HIV- or SIV-infected DCs (17). DC-SIGN has also been recently involved in *Leishmania pifanoi* binding to DCs (18).

Here we show that *M. tuberculosis* infects DCs via ligation of DC-SIGN by the mycobacterial surface-exposed lipoglycan lipoarabinomannan (LAM). Freshly isolated LDCs were found to express DC-SIGN, and *M. tuberculosis*–derived material was detected in DC-$SIGN^+CD14^-HLA$-DR^+ cells in LNs from patients with tuberculosis.

Materials and Methods

Cells and Bacteria. HeLa-derived cells expressing or not DC-SIGN (P4-DC and P4, respectively) (19), were cultured and infected in RPMI-1640 (GIBCO BRL/Invitrogen) supplemented with 10% heat-inactivated FCS (Dutscher). Mononuclear cells were isolated from the blood of healthy volunteers (Etablissement Français du Sang) by Ficoll-Paque centrifugation. T, B, and NK cells were depleted using M-450 Pan T/CD2 and M-450 Pan B/CD19 Dynabeads (Dynal). The recovered cells, referred to as monocytes, were seeded in 6-well plates at 2×10^6 cells/well in 3 ml RPMI-1640, 10% FCS, L-glutamine, granulocyte/Mϕ-colony stimulating factor (10 ng/ml), and interleukin 4 (20 ng/ml; both from R&D Systems). This resulted in DCs after 5 d of culture. Cultures were fed every 2 d with fresh medium containing full doses of cytokines. GFP-expressing strains of *M. tuberculosis* H37Rv and *M. bovis* BCG were generated by transformation with the GFP-encoding plasmid pEGFP and propagated in medium containing 50 μg/ml hygromycin B (Boehringer). Human lung DCs (LDCs) were isolated as described (20, 21). Lung samples were from surgical specimen distant from primary carcinoma, obtained with the patients' consent, and used according to institutional guidelines. In brief, after treating the lung fragments with collagenase, cells were separated on a Ficoll-Paque gradient to obtain pulmonary mononuclear cells, which were cultured in Petri dishes for 1 h before removing nonadherent cells. Adherent cells were further incubated for 16 h in medium. Loosely adherent mononuclear cells released after three rinses in saline were separated into LDCs and autofluorescent alveolar Mϕs with a FACStar™ (Becton Dickinson) according to the presence or absence of autofluorescent inclusions, using a 488 nm wavelength for excitation and a 588 nm filter for emission. Gates were set to remove cell debris and to select LDCs. In contrast to alveolar Mϕs, the latter cells are potent stimulators of allogeneic T lymphocytes (data not depicted, and reference 20). LDCs represented 0.3 to 0.8% of the total cells.

Lymph Node Samples. Lymph nodes were referred to the Laboratory of Pathology at the Saint-Louis Hospital (Paris, France) for the purpose of tuberculosis diagnosis with the patients' consent and used according to institutional guidelines. Human tissues were previously fixed in AFA (Carlo Erba), a mix of 2% formalin (vol/vol), 5% acetic acid (vol/vol), 75% ethanol (vol/vol), and 18% water (vol/vol), and then embedded in paraffin for histopathological diagnosis. Smear positive and/or culture positive (in less than 12 d) biopsies were selected from the collection between May 1995 and December 2001, and ~10 sections per biopsy were used for immunostaining.

Binding Assay. Cells were infected at the indicated multiplicity of infection (MOI) for 4 h at 4°C in RPMI-1640, 10% FCS, extensively washed in RPMI-1640, and analyzed by flow cytometry. Fluorescence was assessed on a total of 2×10^4 cells per sample using a FACSCalibur™ and CELLQuest™ software (Becton Dickinson). In some experiments, the same samples were also plated out onto agar medium and CFUs were scored after 3 wk at 37°C. Alternatively, infections were performed in the presence of 10% complete human serum, in order to opsonize bacteria with complement, as indicated (22).

Antibodies. The following mAbs were used in binding inhibition experiments: anti-CR3/CD11b (clone M1/70; BD Biosciences), -CR3 (2LPM19c; Dako), -MR (clone 15/2; HyCult Biotechnology), -CD40 (clone 5C3; BD Biosciences), and -DC-SIGN (clones 120507; R&D Systems). 1B10 (IgG2a-κ) were produced as follows: Balb/c mice were immunized with 293T cells transfected with cDNA encoding DC-SIGN (cloned from human monocyte-derived DCs). Hybridoma supernatants were screened for the ability to recognize DC-SIGN–expressing HeLa cells, and were purified from bulk cultures. 1B10 neutralizes HIV gp120 binding to DC-SIGN and prevents trans-infection of $CD4^+$ T cells by HIV-pulsed DC-$SIGN^+$ cell lines (A. Amara, personal communication). Binding inhibition experiments were performed by preincubating the cells with the indicated mAbs at different concentrations (see legends to figures) for 1 h at 4°C before the binding assay. For confocal microscopy, DC-SIGN was detected using clone 120507 mAb and a Cy3-conjugated secondary mAb (Amersham Biosciences). For flow cytometry, DC-SIGN, CR3/CD11b, and MR were detected using phycoerythrin-conjugated mAbs from clones 120507, M1/70, and 3.29B1.10 (Immunotech), respectively; CD14 was detected using an allophycocyanin-conjugated mAb (clone M5E2, BD Biosciences); HLA-DR was detected using fluorescein isothiocyanate-conjugated mAb (clone H279; Immunotech). For immunohistochemistry, CD3, CD20, DC-SIGN, CD14, HLA-DR, and *M. tuberculosis* were detected using polyclonal rabbit serum (Dako), mAbs from clone H1(FB1; Dako), from clone 1B10, from clone 7 (Novocastra), from clone CR3/43 (Dako), and an anti–*M. bovis* BCG polyclonal rabbit serum, respectively.

Results and Discussion

Given the unique richness of the mycobacterial envelope in poly-mannosylated materials (23), we asked whether *M. tuberculosis* interacts with DC-SIGN on the surface of DCs. We first compared the binding of virulent GFP-expressing *M. tuberculosis* H37Rv, the most commonly used reference *M. tuberculosis* strain, to HeLa-

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derived cells expressing or not DC-SIGN (P4-DC and P4, respectively; reference 19). We found that *M. tuberculosis* bound to P4-DC cells up to 25 times more than to P4 cells (Fig. 1 A). Similar results were obtained with *M. tuberculosis* clinical isolate MT103 (data not shown), indicating that binding to DC-SIGN was not restricted to laboratory mycobacterial strains. We then studied the binding of *M. tuberculosis* to DCs, and the role of DC-SIGN in this process, as compared with CR3 and MR. As reported (24), human monocyte-derived DCs (MDDCs; reference 25) expressed high levels of DC-SIGN together with CR3 and MR. We performed a binding assay with MDDCs that had been preincubated or not with different mAbs. Preincubation of MDDCs with two different anti–DC-SIGN mAbs inhibited attachment of *M. tuberculosis* up to 90% (Fig. 1 B). Interestingly, preincubation with two anti-CR3 mAbs, used in combination, an anti-MR mAb, or an irrelevant (i.e., directed against a non-mycobacteria-binding protein) anti-CD40 mAb, had only minor effects on the binding of *M. tuberculosis* to MDDCs (Fig. 1 B). Under the same conditions anti-CR3 and -MR mAbs inhibited *M. tuberculosis* binding to monocyte-derived Mϕs (MDMϕs) by ~50 and ~45%, respectively (data not shown), thus confirming previous reports that CRs and MR are major *M. tuberculosis* receptors on MDMϕs (22). It was also important to assess whether DC-SIGN was still the predominant *M. tuberculosis* receptor on DCs in the presence of a complement source, a condition that might be expected in vivo. To this end, we performed a binding inhibition experiment in the presence of complete human serum. Anti-DC-SIGN mAbs were then still able to inhibit up to 90% mycobacterial binding to DCs (Fig. 1 B). Under the same conditions, anti-CR3 and -MR mAbs inhibited mycobacterial binding to MDMϕs by ~60 and ~20%, respectively (data not shown). Thus, our results indicate that DC-SIGN acts as the major *M. tuberculosis* receptor on human MDDCs, even in the presence of complement.

We next examined whether DC-SIGN contributes to the attachment of other intracellular bacterial species to MDDCs. Binding of the vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), which belongs to the tuberculosis complex, was also found to be mediated by DC-SIGN (Fig. 1 C). However, pretreatment with anti–DC-SIGN mAbs had no effect on the binding of either the Gram-positive *Listeria monocytogenes* or the Gram-negative *Salmonella typhimurium* species (Fig. 1 C). Major structural differences exist at the surface of mycobacteria compared with $Gram^+$ and $Gram^-$ species, which may explain differences in binding to DC-SIGN. In particular, LAM is an abundant poly-mannosylated lipoglycan, specific to the mycobacterial envelope (26), and it has been shown to bind to various human C-type lectins on Mϕs, such as surfactant protein D (27). Inasmuch as DC-SIGN is a C-type lectin that recognizes mannose-rich molecules (16), we investigated whether *M. tuberculosis* binding to the lectin was mannosyl-defined. Binding of *M. tuberculosis* to both P4-DC and MDDCs was inhibited up to 90% by yeast mannan as well as by *M. tuberculosis* H37Rv-derived LAM (Fig. 1

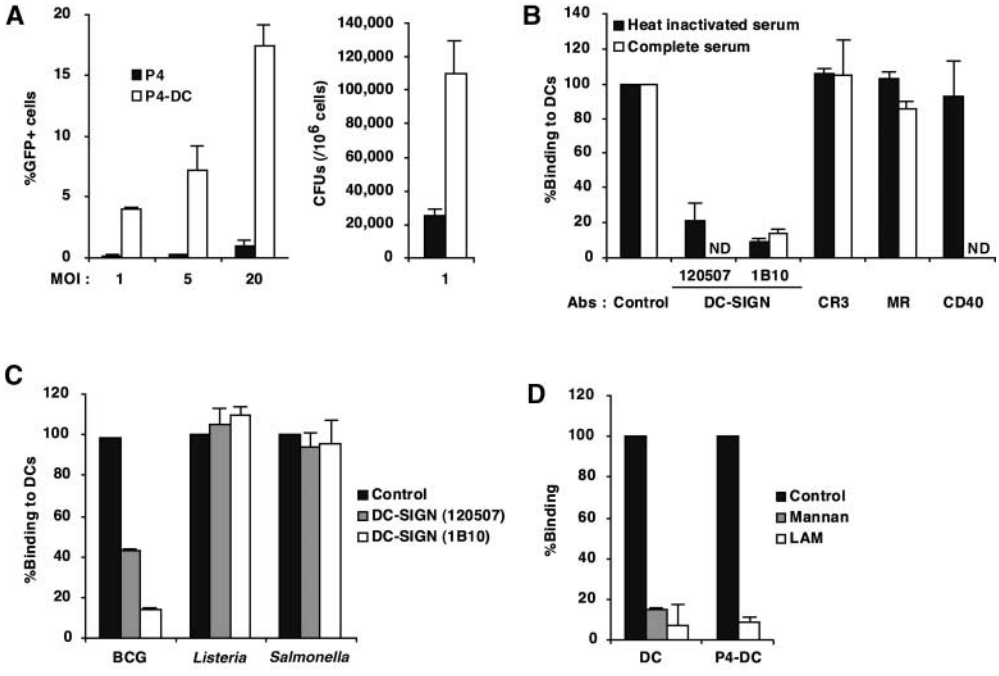

Figure 1. *M. tuberculosis* binds to DC-SIGN. (A) Epithelial HeLa-derived P4 cells expressing or not DC-SIGN (P4-DC and P4, respectively) were infected with GFP expressing (GFP+) *M. tuberculosis* H37Rv at different MOIs. Bacteria binding was evaluated by flow cytometry (left panel) and CFU counts (right panel). Data represent means (±SD) of three separate experiments. (B) MDDCs were infected with GFP-*M. tuberculosis* H37Rv at an MOI of 1 bacterium per cell in the presence or not of 10% complete human serum, either directly (control) or after preincubation with 10 μg/ml of mAbs directed against CR3/CD11b, MR, CD40, or DC-SIGN. Bacteria binding was assessed by flow cytometry. Preincubation with the corresponding isotype controls led to no significant inhibition of mycobacteria binding (not shown). Data were expressed as percentages of binding relative to control values (100%, no mAb), and means (±SD) of three independent experiments are shown. (C) MDDCs were infected with GFP-*M. bovis* BCG, *Salmonella typhimurium* (clinical isolate), or *Listeria monocytogenes* (clinical isolate) and subjected to the binding assay. In experiments using *S. typhimurium* and *L. monocytogenes*, infected cells were plated out onto agar medium and CFUs were scored after 24 h at 37°C. Data are expressed as in B. (D) *M. tuberculosis* binding to DC-SIGN is inhibited by LAM. Cells were pretreated for 1 h at 4°C with 10 μg/ml mannan as control, or with 10 μg/ml LAM, and subjected to the binding assay. Data are expressed as in B.

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D). By contrast, preincubation of cells with LPS derived from *Escherichia coli* or with dextran had no effect on the binding process (data not shown). These findings suggest that LAM may constitute a privileged mycobacterial ligand for DC-SIGN, even though other mycobacterial components may also bind to the lectin. Interestingly, *Mycobacterium smegmatis*–derived LAM, that is devoid of mannose capping residues, was found to only moderately inhibit *M. tuberculosis* binding to DC-SIGN (unpublished data).

After attaching to the cell surface, pathogenic mycobacteria are taken up by phagocytic cells and reside in phagosomes that do not fuse with host cell late endosomes and lysosomes, but take part in the recycling pathway (1). To investigate DC-SIGN trafficking in *M. tuberculosis*-infected cells, we performed confocal microscopy analysis of MDDCs infected with GFP-expressing mycobacteria. During the first hour of phagocytosis, most bacilli were detected as either attached extracellularly to the cells (Fig. 2 A, top panel), or colocalized with DC-SIGN in nascent phagosomes (Fig. 2 A, middle panel). However, DC-SIGN staining was not detected on phagosomes that had detached from the plasma membrane, indicating that it was excluded from the vacuoles very soon after phagocytosis (Fig. 2 A, bottom panel). These data indicate that DC-SIGN is present in *M. tuberculosis* vacuoles during the early steps of bacterial uptake, and is then rapidly expelled from the phagosome (Fig. 2 B), possibly as a result of recycling to the cell plasma membrane. We also examined whether *M. tuberculosis* infection could modify DC-SIGN expression at the surface of infected cells. As reported (6, 7), infection was found to induce cell maturation, as illustrated by upregulation of CD83 (Fig. 2 C), CD86, and HLA-DR (data not shown). DC-SIGN expression was only slightly downmodulated in mature infected cells, even 48 h after infection (Fig. 2 C).

It was then important to determine whether *M. tuberculosis* could interact with DC-SIGN$^+$ cells in vivo. We first evaluated the presence of the lectin on human interstitial LDCs as compared with in vitro–generated MDDCs. LDCs were isolated from surgical specimens in tissues distant from limited primary lung carcinomas. As reported (20), all LDCs were HLA-DR$^+$ (Fig. 3 A) and CD14$^-$ (data not shown), a phenotype shared by MDDCs. Like MDDCs, LDCs expressed surface DC-SIGN, CR3, and MR (Fig. 3 A). Although we cannot formally rule out the possibility that LDC preparations were devoid of contaminant cells of other type(s) (e.g., activated macrophages), it is tempting to suggest that *M. tuberculosis* may encounter and interact with DC-SIGN$^+$ DCs during the natural course of infection. However, too few cells could be recovered from surgical samples to allow us to perform binding experiments to test this hypothesis. To further investigate DC-

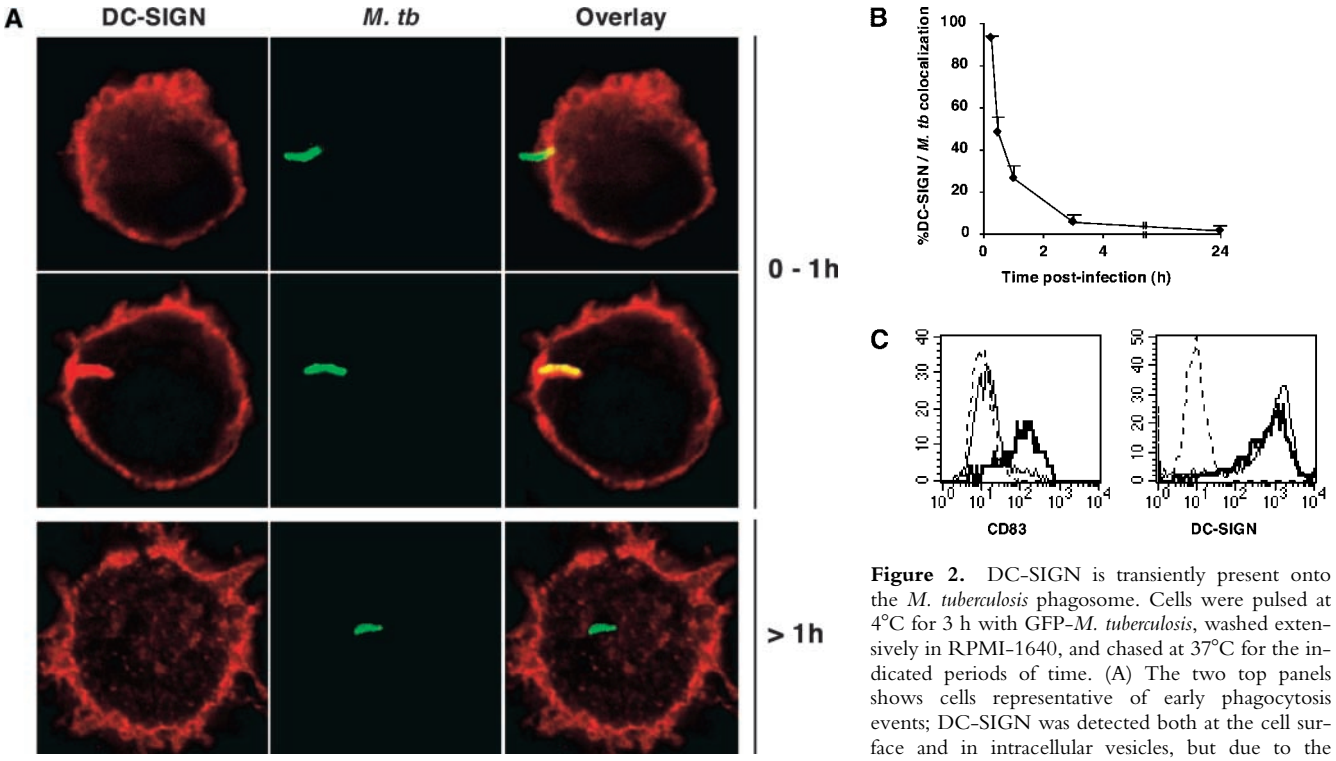

Figure 2. DC-SIGN is transiently present onto the *M. tuberculosis* phagosome. Cells were pulsed at 4°C for 3 h with GFP-*M. tuberculosis*, washed extensively in RPMI-1640, and chased at 37°C for the indicated periods of time. (A) The two top panels shows cells representative of early phagocytosis events; DC-SIGN was detected both at the cell surface and in intracellular vesicles, but due to the strong surface staining, the red signal had to be reduced. Each panel shows a representative cell. (B) Kinetics of DC-SIGN colocalization with *M. tuberculosis* (*M. tb*): a minimum of 100 bacteria were scored at each time point. Extracellular bacteria that were found attached to the cells (early time points, 15 and 30 min) were scored as colocalizing with DC-SIGN. Results are means (±SD) of three separate experiments. (C) Surface expression of CD83 (left panel) and DC-SIGN (right panel) on MDDCs was assessed by flow cytometry 48 h after infection with GFP-*M. tuberculosis* H37Rv. For analysis, cells were gated on GFP-*M. tuberculosis*–infected MDDCs. Dotted line: isotype control labeling; plain lines: uninfected cells; bold lines: infected cells.

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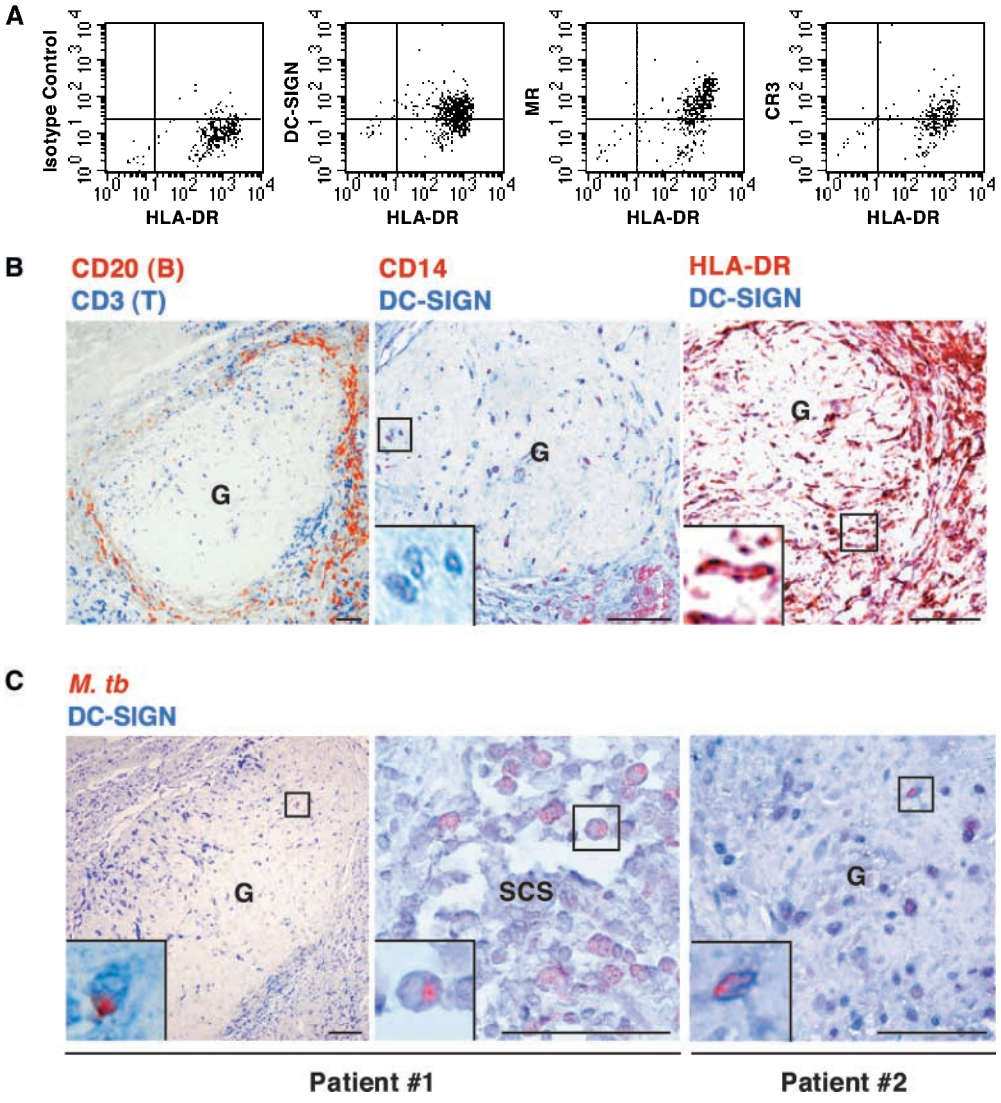

Figure 3. DC-SIGN expression on lung DCs (LDCs) and in lymph nodes (LNs). (A) LDCs are HLA-DR$^+$ and express DC-SIGN, CR3, and MR. Surface expression of HLA-DR, DC-SIGN, CR3/CD11b, and MR on LDCs from a noninfected patient was assessed by flow cytometry using the appropriate cytochrome-conjugated mAbs. (B) DC-SIGN expression in the LN from a patient with tuberculosis (G, granuloma). Left panel, CD3 (blue) and CD20 (red); middle panel, DC-SIGN (blue) and CD14 (red); right panel, DC-SIGN (blue) and HLA-DR (red). (C) Localization of *M. tuberculosis*–derived antigens in DC-SIGN$^+$ cells in LNs from two patients with tuberculosis. DC-SIGN (blue) and *M. tuberculosis* (red) were immunodetected both in granulomas (G; left and right panels) and in nongranulomatous regions, including subcapsular sinuses (SCS; middle panel). In B and C, bars represent 0.5 mm and squares represent areas shown at higher magnification at the single cell level in the insets. Staining of the samples with IgG2a (1B10 isotype control) or with a naive rabbit serum led to no detectable signal (data not depicted).

SIGN possible involvement in the interactions of *M. tuberculosis* with DCs in vivo, we reasoned that if DC-SIGN$^+$ DCs take up *M. tuberculosis* in the lungs, and since DC-SIGN expression is only slightly reduced by infection-associated maturation of the cells, then we should be able to detect mycobacteria-derived material in DC-SIGN$^+$ DCs in LNs from patients with tuberculosis. LN paraffin-embedded sections from seven patients were stained with anti-mycobacterial and anti–DC-SIGN mAbs (Fig. 3 B). Samples containing viable bacilli were selected based on smear and culture positivity. The selected LNs were rich in granulomas, characterized by caseous centers surrounded by rings of T and B lymphocytes (Fig. 3 B, left panel). Granulomas are typical of mycobacterial infections. They develop after recruitment and accumulation of effector lymphocytes around infected foci. As reported (15, 28), DC-SIGN was detected mostly in intergerminal T cell zones and not in germinal centers. In addition, DC-SIGN$^+$ cells were located in granulomatous structures, and were CD14$^-$ and HLA-DR$^+$ (Fig. 3 B, middle and right panels, respectively). Mycobacteria and mycobacteria-derived antigens were immunodetected in samples from 5 out of 7 patients, in both granulomatous (Fig. 3 C, left and right panels), and nongranulomatous regions of the LNs, including subcapsular sinuses (Fig. 3 C, middle panel). In most cases (~80–100%), mycobacteria-specific signal was detected within DC-SIGN$^+$ cells (Fig. 3 C). These findings indicate that *M. tuberculosis* interacts with DC-SIGN in vivo and that DC-SIGN$^+$ cells, possibly DCs, may carry mycobacteria or mycobacteria-derived material from the lungs to the LNs during their maturation process.

Altogether, our results demonstrate that DC-SIGN/CD209 is the predominant *M. tuberculosis* receptor on human DCs, whereas the mycobacterial Mϕ receptors, CR3 and MR, appear to play a minor role, if any, in this binding. This exclusivity may be due to DC-SIGN abundance on DCs relative to CR3 and MR, and/or to the affinity of the lectin for its ligand(s). Affinity for DC-SIGN seems to be fairly restrictive among bacteria, as neither Gram$^+$ *L. monocytogenes* nor Gram$^-$ *S. typhimurium* species bound to

the lectin. This is not surprising, as the envelopes of Gram$^+$ and Gram$^-$ bacteria are very poor in poly-mannosylated material. Unique characteristics of the mycobacterial cell wall, which is the most complex of all bacterial cell surfaces (23), might thus account for the affinity for DC-SIGN. Our finding that LAM, like mannan, which contain common mannosyl motifs, can block DC-SIGN–mediated attachment of *M. tuberculosis* to DCs and to P4-DC cells is consistent with the high affinity of the lectin for mannose-rich molecules (16), and suggests that LAM may be one of its major mycobacterial ligands.

Ligation of DC-SIGN by mycobacteria is likely to have important effects on the immunological and pathological events associated with *M. tuberculosis* infection. Differential receptor usage by *M. tuberculosis* on DCs and Mϕs may account for the different survival ability and trafficking patterns of mycobacteria in the two cell types, which is still a matter of debate (7, 9, 10). DC-SIGN has been detected on alveolar Mϕs (28, 29), that constitute the privileged cell targets of *M. tuberculosis* during the early steps of infection. It will be important to evaluate whether the lectin is also a predominant mycobacterial receptor on this cell population. It will also be of great interest to investigate what type of pro- or antiinflammatory cytokines are induced or repressed upon DC-SIGN ligation by mycobacteria (30), as compared with ligation of other signal transducers, such as the TLRs (14). DCs are also involved in the early activation of non-MHC–restricted and CD1-restricted T cells specific for various mycobacterial glycolipids, including LAM (31). The intracellular trafficking pattern of DC-SIGN in *M. tuberculosis*-infected DCs suggests that DC-SIGN may carry mycobacterial glycolipids from the bacterial vacuole to the cell plasma membrane and/or to various subcellular compartments, where glycolipids could be loaded onto CD1 molecules for presentation to CD1-restricted lymphocytes (32).

In the lungs, submucosal and interstitial LDCs are thought to play a key role in immune surveillance of the respiratory tract (5). In particular, interactions of DCs present in the alveolar septal walls with *M. tuberculosis* could be crucial for initiating an efficient anti-mycobacterial immune response. Our finding that LDCs express DC-SIGN suggests that DC-SIGN is likely to interact with *M. tuberculosis* in vivo. This is strengthened by detection of mycobacteria-derived material in DC-SIGN$^+$ DCs in LNs from patients with tuberculosis. It is interesting that both *M. tuberculosis* and HIV can bind to DC-SIGN. DC-SIGN–expressing cells may carry either intracellular or surface-attached *M. tuberculosis* during their migration from the site of infection to the LNs, and could thus constitute a mycobacterial reservoir. This has also been suggested for HIV (17), and might account for several pathological and immunological aspects of *M. tuberculosis* infection, e.g., mediastinal adenitis, the formation of secondary granulomas in the LNs, and the chronic stimulation of the immune system that is required to maintain the latency period of the disease.

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